COMPARISON OF DEMINERALIZED DENTIN AND DEMINERALIZED FREEZE DRIED BONE AS CARRIERS FOR ENAMEL MATRIX PROTEINS IN A RAT CRITICAL SIZE DEFECT

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Kerri Font, D.D.S.

The University of Texas Graduate School of Biomedical Sciences at San Antonio

Supervising Professor: David L. Cochran

This study evaluated the ability of Emdogain (EMD) in combination with demineralized freeze dried bone (DFDBA) or demineralized dentin matrix (DDM) to enhance bone regeneration as well as the ability of these carriers to maintain EMD at the surgical site. Critical size defects were created in rat calvaria. In Part I of the experiments,

varying amounts of EMD (1 mg, 3 mg, 9 mg) with DFDBA or DDM were placed in the defects. DFDBA, DDM, EMD alone, and propylene glycol served as controls (8 rats/group). Rats were euthanized at two and eight weeks. Histological analysis was used to score for the presence of carrier material and new bone formation. The data were analyzed using RIDIT analysis ($p \leq 0.05$). In Part II of the experiments, EMD was labeled with ^{125}I and placed in the defect alone or combined with DFDBA or DDM particles. Images were acquired using gamma scintigraphy at 0, 4 hour, 1, 2, 3, 6, 13, 20 days. The images were quantified using computer software to determine the retention of EMD in the critical size defect.

At two weeks there was no statistically significant difference between the groups for the amount of new bone formation. At eight weeks there was no statistically significant difference in the new bone formation between the experimental groups with the exception of 3 mg EMD in combination with DDM particles, which showed statistically significant new bone formation ($p=0.0069$). Unlike DFDBA, new bone was not present on the surface of the DDM particles. The new bone formed around the DFDBA particles was in intimate contact with the particle whereas; soft tissue encapsulated the DDM particles. Though this encapsulation was unexpected, it did not appear to interfere with the new bone formation. The ^{125}I labeled EMD disappeared rapidly from the defect with approximately 50% of the radioactivity remaining at 4 hours, 20% at 24 hours, and 4% on day 20. Neither carrier increased the longevity of EMD in the defect. These results indicate that while placement of DFDBA and DDM does not enhance new bone formation with EMD compared to EMD alone, they do not interfere with it either.

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A
THESIS

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Kerri Font, D.D.S.

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THE VIEWS EXPRESSED IN THIS ARTICLE ARE THOSE OF THE AUTHOR AND DO NOT REFLECT THE OFFICIAL POLICY OR POSITION OF THE UNITED STATES AIR FORCE, DEPARTMENT OF DEFENSE, OR THE U.S. GOVERNMENT.

DEDICATION

This thesis dedicated to my husband [redacted], who is everything I am not and everything I would like to be. I also would like to thank my mom and sister for all the support and encouragement. Without you three I would not be where I am today.

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INTRODUCTION AND LITERATURE REVIEW

A. Introduction

Periodontal disease may result in destruction of periodontal attachment and eventual loss of bone and/or teeth. About 13% of the population suffers from the moderate to severe forms of this disease (Albander *et al* 1999). The initial step in treating periodontal disease includes closed mechanical cleaning of the pockets. The goal is to decrease the risk of disease progression and establish a healthy periodontium. Once the disease progression has stabilized, the ideal treatment would include regeneration of periodontal attachment and bone. Until recently, regenerative procedures were not considered predictable and pocket reduction through a resective approach was commonly used to maintain long term periodontal health. Treatment modalities that may promote regeneration now include alveolar bone grafting, guided tissue regeneration, and the use of growth factors such as those present in platelets. Stimuli are needed for the regeneration of cementum, bone, and periodontal ligament which make up the periodontal apparatus. This regeneration requires the coordination of a cascade of events that may ultimately result in integrated tissue formation and include different cell types interacting at specific times with a multitude of growth factors, hormones, and the extracellular matrix. Alveolar bone grafting, guided tissue regeneration and growth factors have been shown to regenerate parts of the periodontal attachment apparatus in humans (Bowers *et al* 1989b, Sculean *et al* 1999). To increase the amount and predictability of regeneration many different biological mediators have been

evaluated for their ability to stimulate reattachment and bone growth. Some of these include differentiating polypeptides, growth factors, and extracellular matrix proteins (Cochran, Wozney 1999). These biological mediators are naturally occurring hormones or proteins that are purified and used in vivo in higher concentrations than would occur naturally. Enamel matrix proteins (EMD), derived from porcine tooth germs as an extracellular matrix, may stimulate periodontal regeneration by mechanisms that differ from previous grafting techniques. Theoretically, EMD is able to regenerate the periodontal apparatus by stimulating the same cells that are active in the bell stage during tooth development when the tooth supporting apparatus is formed (Hammarstrom 1997).

B. Odontogenesis

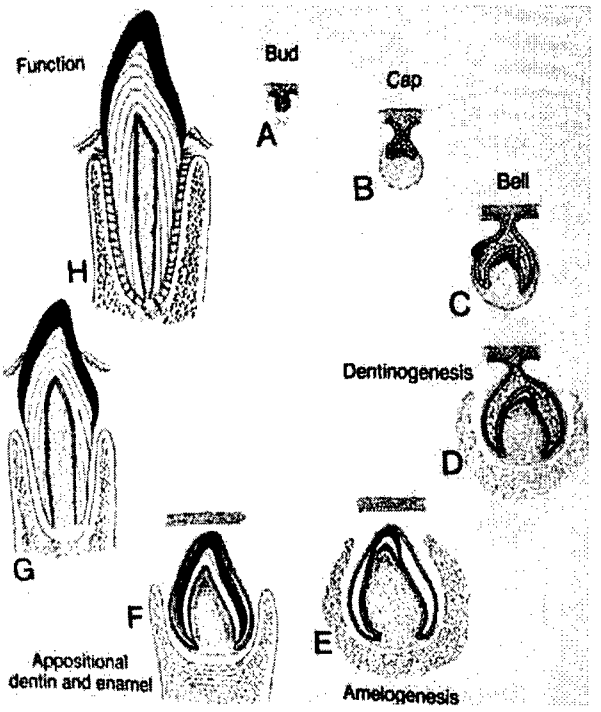
Human odontogenesis begins in the sixth week of gestation. The epithelial component begins releasing transcription factors to turn on the ectomesenchymal cells. These ectomesenchymal cells have the capacity to induce epithelial cells to differentiate into ameloblasts. These enamel-producing cells subsequently stimulate the mesenchymal cells to differentiate into odontoblasts. As teeth develop they pass through three stages of growth: bud, cap, and bell. These stages are followed by apposition, root formation, and eruption (Figure 1).

In the bud stage epithelial structures and the dental lamina are formed. The cap stage results in the epithelial derived enamel organ and the ectomesenchymal derived dental papilla. The dental pulp and dentin are formed from the dental papilla. The dental follicle, which gives rise to the future periodontal ligament, alveolus, and cementum,

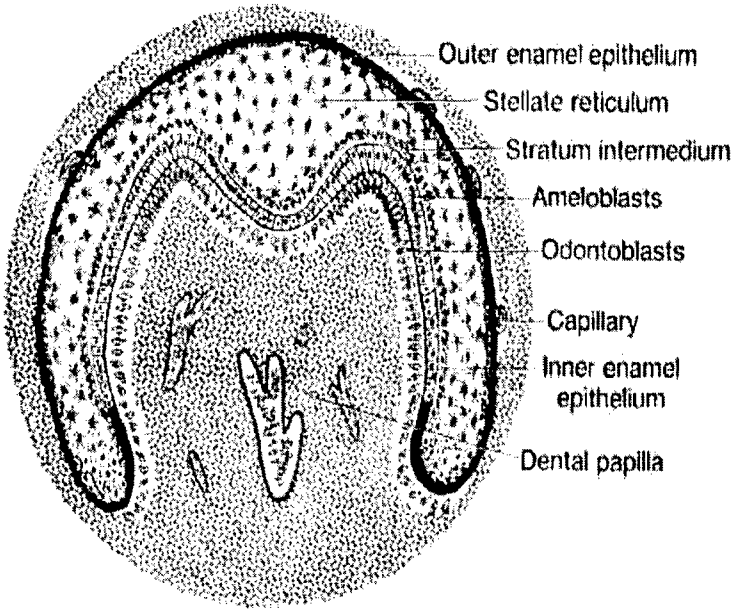
Figure 1: Stages of Tooth Development

Slide A: As teeth develop they pass through three stages of growth: bud, cap, and bell. These stages are followed by apposition, root formation, and eruption.

Slide B: Initial dentinogenesis, cells of the dental papilla stimulate cells of the enamel organ to express signaling molecules such as BMP-2, BMP-4, BMP-7.



A.



B.

Oral Development and Histology Second Edition; Avery J. 1994

surrounds the entire cap. The bell stage is characterized by further histodifferentiation of the cells. Cells of the dental papilla stimulate cells of the enamel organ to express signaling molecules such as BMP-2, BMP-4, BMP-7, sonic hedgehog, and fibroblast growth factor-4 (Gartner 1999). Immediately following the formation of the dentin matrix, the ameloblasts produce enamel matrix. Root formation starts as dentinogenesis and amelogenesis approach the cervical loop. The inner and outer enamel epitheliums join to make the Hertwig's epithelial root sheath. Hertwig's epithelial root sheath elongates until the rim folds in medially. As the Hertwig's epithelial root sheath breaks down, cells of the dental sac migrate to become cementoblasts (Gartner 1999). Cells from the enamel organ differentiate into the enamel matrix. Some of the signaling molecules coming from the enamel matrix are BMP-2, insulin–like growth factor, and transforming growth factor-β. These signaling molecules initiate the differentiation of the dental papilla that will produce the dentin matrix (Gartner 1999).

Knowledge of the embryologic development of human teeth stimulated interest in mimicking normal development in order to regenerate the periodontal apparatus. Due to the cellular differentiation that occurs during the bell stage, the course of this process became the focus of research. A new paradigm in periodontal regeneration began to form that lead researchers to examine modulating natural growth factors as a way to selectively increase cellular differentiation. As with many of the graft materials in periodontics, animals served as a source for a developmental protein.

EMD is freeze dried porcine enamel matrix protein that is harvested from the developing teeth germs of six month old piglets (Hammarstrom 1997). EMD contains some of the same proteins that are found in immature enamel. Ninety percent of the

composition of EMD is amelogenins and its breakdown products. Other proteins found in EMD are proline-rich non-amelogenins, enamelin, tuft proteins, tuftelin, and serum proteins. Since EMD is derived from enamel matrix it is thought to primarily stimulate cementoblast development, but it has also been shown to increase proliferation, protein synthesis, and mineralization of periodontal ligament cells (Hammarstrom 1997). Enamel matrix proteins are highly conserved and are similar among mammalian species and have been shown to be safe when used with human (Brookes *et al* 1995).

C. Periodontal Regeneration

Periodontal regeneration is the ultimate goal of treatment for restoring and maintaining health in periodontally diseased patients. Initially, this was an unobtainable goal. Histological analysis of treated sites revealed that the majority of healing occurred by long junctional epithelium (Caton *et al* 1980). Melcher, in 1976, introduced the theory of guided tissue regeneration. Melcher hypothesized that the periodontal ligament was the only source of progenitor cells for regeneration. Aukhil *et al*, in 1986, showed that progenitor cells of the PDL could differentiate into cementoblasts. Melcher later revised his theory to include bone as a source for progenitor cells (Melcher *et al* 1987). In 1988, Iglhaut *et al* demonstrated that by excluding the epithelium and gingival connective tissue, both bone and PDL can serve as sources of progenitor cells for regeneration. Surgical techniques started to evolve with the knowledge that excluding the epithelial migration was important for more predictable regeneration.

The pioneer of the epithelial exclusion technique for regeneration was Prichard in 1977. This technique involved removal of overlying gingival tissue leaving interdental bone denuded (Prichard 1977). In 1983, he successfully used this technique to regenerate intrabony defects. Though Prichard was successful with this technique, it was not universally predictable. In the early 80's it became common practice to use non-resorbable membranes to achieve guided tissue regeneration (GTR). Nyman *et al* (1982) was the first to demonstrate that new attachment was possible with GTR in a human model. These membranes were used to exclude the epithelium, thereby ensuring preferential regeneration by osteoblasts and fibroblasts (Minabe 1991). Even with the exclusion of the epithelium, complete regeneration was not always achieved. Therefore, the need to enhance the patient's own regenerative capabilities was hypothesized. In the 1990's bone grafts were combined with the GTR technique.

1. Demineralized freeze dried bone

The gold standard of bone grafts is the patient's own bone. Autografts have both osteogenic and osteoinductive properties. A bone graft is considered osteoinductive if it contains proteins which will induce cells to differentiate into osteoblasts. The drawbacks of autografts include, not having enough bone graft material and in some cases requiring a second site surgery which increases the chance for infection and post operative morbidity. Therefore, clinicians began to look at other sources for bone graft materials. One source is human demineralized freeze dried bone allograft (DFDBA). It has been shown to be a viable osteoinductive graft alternative to an autograft. Studies by Bowers *et al* (1989 b, c) indicate that DFDBA stimulates regeneration of cementum, PDL, as well

as alveolar bone in humans. Once placed into the defect, DFDBA stays in the area and is surrounded by host bone (Becker *et al* 1994). The residual DFDBA particles provide wound stability and maintain space (Reynolds, Bowers 1996). Bone morphogenic proteins are highly conserved proteins in the bone. These proteins have been shown to stimulate osteoinduction (Urist *et al* 1983, Wang, Glimcher 1990, Wozney 1995). Shigeyama *et al* (1995) isolated BMP-2, BMP-4, and BMP-7 from DFDBA. However, studies performed by Schwartz *et al* (1996) have shown that the osteoinductivity varies between DFDBA batches. Age is a major determining factor for the osteoinductive properties (Schwartz *et al* 1998a). Therefore, some batches of DFDBA may be more osteoconductive than osteoinductive. The addition of EMD to active DFDBA has been shown to promote an increase in osteoinductivity compared to the active DFDBA alone (Boyan *et al* 2000). Due to the demonstrated ability of DFDBA to stimulate regeneration, it has been considered to be a good choice as a carrier for EMD.

2. Demineralized dentin matrix

Porcine demineralized dentin matrix derivative (DDM) is the residual product from the production of enamel matrix protein. Demineralized dentin has been shown to induce ectopic bone formation in rats (Bang, Urist 1967, Somerman *et al* 1987). Structurally demineralized dentin is more cross-linked than bone; it has a highly porous structure with many channels that permit rapid diffusion of reagents (Veis, Perry 1967). Chemically demineralized bone and dentin have similar compositions (Bang, Urist 1967). However, demineralized dentin has dentinal proteins, such as phosphophoryns, which are rich in serine, aspartic acid and phosphorous (Dimuzio, Veis 1978). Demineralized bone

proteins contain more glutamic acid than aspartic acid and are lower in serine. Bone is not as highly phosphorylated as dentin and the long acidic sequences in bone do not contain phophorylation sites which could bind calcium ions (George *et al* 1993). Phosphophoryn molecules move directly to the mineralization front and are directly associated with mineralized collagen fibrils (Veis 1993). Similar to demineralized bone, demineralized dentin contains bone morphogenic proteins. Unlike demineralized bone, the bone morphogenic proteins in dentin have not been isolated (Rutherford, Fizgerald 1995). However, both demineralized bone matrix and demineralized dentin matrix contain transforming growth factor-β_1 (TGF-β_1) and insulin-like growth factors (IGF-I and –II) (Finkelman *et al* 1990).

Carvalho *et al* (2004) examined bone formation in critical size bone defects in the mandibles of rabbits. He demonstrated that the addition of demineralized dentin matrix slices with a polytetrafluorethylene membrane accelerated new bone formation in the rabbit mandible. Due to the similarities of demineralized dentin to demineralized bone and the ready availability of demineralized dentin as a byproduct of EMD production, demineralized dentin matrix may be a potential carrier for EMD.

3. Enamel matrix derivative

EMD contains some of the same proteins that are found in immature enamel. 90% of the composition of EMD is amelogenins. Other proteins found in EMD are proline-rich non-amelogenins, enamelin, tuft proteins, tuftelin, and serum proteins. EMD can stimulate acellular cementum formation (Hammarstrom 1997) and enhanced periodontal ligament cell proliferation and attachment (Gestrelius, Andersson 1997, Lyngstradaas *et*

al 2001, Hoang *et al* 2002). In addition research on effects of EMD on osteoblast progenitor cells and osteoblasts suggested that EMD may interact with known regulators, such as bone morphogenic proteins to impact bone regeneration (Iwata *et al* 2002). EMD has been shown to enhance periodontal ligament regeneration and promote healing in defects compared to surgical debridement alone (Heijl 1997, Okdua *et al* 2000, Tonetti *et al* 2002). EMD has been shown to selectively enhance proliferation of periodontal ligament cells, but not epithelial cells (Gestrelius, Andersson 1997). The extracellular matrix proteins are thought to suppress the apical growth of junctional epithelium on root surfaces (Kawase *et al* 2000). EMD produces similar clinical results compared to the clinical results of traditional GTR procedures (Pontoriero *et al* 1999, Silvestri *et al* 2000, Sculean *et al* 2001, Zucchelli *et al* 2002, Sanz *et al* 2004). However, a recent study suggested that EMD may produce a greater reduction of horizontal furcation depth compared to similar sites treated with GTR (Jepsen *et al* 2004). Histological evaluation of intrabony defects indicates that EMD regenerates osseous defects around teeth (Sculean *et al* 2000, Cochran *et al* 2003, Sculean *et al* 2004) and has the greatest affects in smaller sized defects (Cochran *et al* 2003, Silvestri *et al* 2003). A five year follow up study from Sculean *et al* (2004) indicated that the clinical results obtained from EMD were stable up to five years. To date no longer term studies have yet to be published.

Reports show that EMD accelerates soft tissue healing (Francetti *et al* 2004, Hagewald *et al* 2004) by a proposed mechanism including accelerated angiogenesis (Yuan 2003). Finally EMD does not appear to enhance root coverage over traditional connective tissue graft in mucogingival procedures (Modica *et al* 2000, Hagewald *et al* 2002, McGuire, Nunn 2003).

Propylene glycol alginate is used as carrier for freeze-dried EMD in clinical procedures. Newer formulations supply the EMD in a gel solution containing 30 mg/ml of EMD in a syringe containing 0.3 ml for a single defect or 0.7 ml for three periodontal defects. Possibly because EMD carrier has antimicrobial properties (Sculean *et al* 2001, Newman *et al* 2003) and unlike other grafting agents, no benefit of postoperative antibiotics has been shown in treatments using EMD (Sculean *et al* 2001). So far, EMD has been shown to be safe in multiple surgical procedures (Heard *et al* 2000). The body views EMD as a "self protein" and does not produce allergic or other adverse effects after clinical treatments (Froum *et al* 2004). This is due to the similarity in amino acid sequence in pigs and humans indicating these proteins are highly conserved over time.

However, propylene glycol alginate has some inherent characteristics that are less than ideal for a carrier of EMD. First propylene glycol has a low viscosity after rising to body temperature and therefore makes the clinical handling of EMD a challenge. The low viscosity of the material allows for movement outside the defect area presumably after the proteins have precipitated in the lesion. Second, the carrier does not maintain physical space with the potential for less defect fill. Multiple studies have evaluated using a xenograft carrier in combination with EMD. The combination of graft material and EMD consistently produced better clinical parameters (Lekovic *et al* 2000, Zucchelli *et al* 2003). DFDBA in combination with EMD was shown to have similar soft tissue healing as EMD alone; but the addition of DFDBA increased the osseous fill (Gurinsky *et al* 2004). This investigation speculated that combining particulate graft material with EMD would maintain EMD in the periodontal defect.

4. Rat Calvaria Model

A critical size defect is a defect that will not heal during the lifetime of the animal. Testing bone regenerative materials in a critical size defects allows the osteogenic potential of the material to be considered unequivocal. In the rat calvaria model the critical size defect is made in the parietal bone between the frontal and occipital bones. The calvarium is a pure membranous bone that normally heals by intramembranous ossification. The main reasons why calvarial defects do not heal spontaneously in adults are because there is very little bone marrow and the blood supply is poor (Schimtz, Hollinger 1985). The first model for a rat critical size defect was 2 mm in which the defect reportedly failed to heal in 12 weeks (Turnbull, Freeman 1974). However, in 1982, Tagaki and Urist demonstrated that an 8 mm defect created in the calvarium resulted in 3 mm of spontaneous healing with the remaining 5 mm healing with fibrous connective tissue. Currently, 8 mm diameter defects are considered critical size in the rat calvarium and are used for the testing of bone regenerative materials.

D. Statement of the Problem and Objectives

EMD has been in clinical use since 1997 and several studies have found it to have significant but inconsistent regenerative capabilities particularly in larger defects (Yukna, Mellonig 2000, Parodi *et al* 2004). Since the low viscosity of the EMD carrier allows it to flow away from the defect site, suturing techniques have been employed to retain the material at the site. Although there is some evidence that EMD can regenerate one wall defects, the absence of space maintenance properties of the EMD with the present carrier

may be one reason for the inconsistent regeneration observed. One way to maintain the space in a defect and keep EMD localized to the defect would be to use a different carrier substance with EMD.

Therefore, the objective of this study was to evaluate two carrier substances, DFDBA and DDM, to potentially enhance the response of EMD and to test whether EMD placed with DFDBA or DDM was retained at the surgical site. Our hypothesis was that the addition of a carrier to EMD would increase the duration of EMD in the defect site and thereby increase bone formation. The following specific aims were established to fulfill this objective:

1) To evaluate the ability of EMD in combination with DFDBA or DDM to enhance bone regeneration.
2) To evaluate the ability of DFDBA or DDM to retain EMD at the surgical site.

MATERIALS AND METHODS

A. Xenografts

Human DFDBA (Musculoskeletal Transplant Foundation, Dentsply Ceramed Dental Lot MTF 006213930502) ranging in size from 250-860 μm in diameter was obtained in the clinical use form. Porcine DDM (XP40 Lot 0102) was obtained from Biora AB Malmo, Sweden (Biora was acquired by Straumann (Basel, Switzerland) in 2004). These particles were of a much larger size, ranging from 1000-4100 μm. The DFDBA and DDM were tested in an 8 mm critical size defect to assess bone induction ability.

B. Enamel Matrix Derivative

Porcine embryonic enamel matrix derivative was obtained from the manufacturer (Biora AB Malmo, Sweden) in the lyophilized form available for clinical use. It was reconstituted with propylene glycol alginate (diluent) (Biora AB Malmo, Sweden, Lot PGA2113).

C. Analysis of New Bone Formation

1. Preparation for Implants

DFDBA (10 mg) and DDM (10 mg) particles were placed into sterile tubes. When the EMD was indicated 1, 3, or 9 mg of EMD with propylene glycol carrier was added to the DFDBA or DDM particles in the tubes, or placed in a separate sterile tube. On the day of implantation the contents of the tube were placed in a sterile dampen dish and the diluent (24 μl propylene glycol alginate) was added and mixed with a sterile spatula.

2. Animal Protocol

Sprague-Dawley (Charles River) rats 70-85 days / 300-349 grams were used for this study. The Institutional Animal Care and Use Committee at UTHSCSA approved this protocol prior to commencement of the study.

3. Experimental groups

The rats were divided into 14 groups per time point (2 or 8 weeks) with 8 rats in each group (Tables 1): 1) diluent, 2) 1 mg EMD with diluent, 3) 3 mg EMD with diluent, 4) 9 mg EMD with diluent, 5) DFDBA and diluent, 6) DFDBA without diluent, 7) 1 mg EMD with diluent and DFDBA, 8) 3 mg EMD with diluent and DFDBA, 9) 9 mg EMD with diluent and DFDBA, 10) DDM and diluent, 11) DDM without diluent, 12) 1 mg EMD with diluent and DDM, 13) 3 mg EMD with diluent and DDM, 14) 9 mg EMD with diluent and DDM.

Table 1: Experimental Groups

The following table illustrates the division of rat groups. There were 14 groups at each time point (two and eight weeks) with eight rats per group.

	2 weeks	8 weeks
Diluent	**Group 1**	**Group 15**
Diluent+1, 3, 9 mg EMD	**Group 2, 3, 4**	**Group 16, 17, 18**
DFDBA+diluent	**Group 5**	**Group 19**
DFDBA without diluent	**Group 6**	**Group 20**
DFDBA+1, 3, 9 mg EMD	**Group 7, 8, 9**	**Group 21, 22, 23**
DDM+diluent	**Group 10**	**Group 24**
DDM without diluent	**Group 11**	**Group 25**
DDM+1, 3, 9 mg EMD	**Group 12, 13, 14**	**Group 26, 27, 28**

4. Surgical Protocol

The rats were anesthetized by intramuscular injection. The anesthesia cocktail contained 6 ml Ketamine/Acepromazine (1 ml Acepromazine (100 mg/ml) and 10 ml Ketamine (100 mg/ml)) and 4 ml small animal Rompur (20 mg/ml). Subsequently rats were maintained on isoflurane gas (4 L/min) with oxygen (2 L/min). The head of each rat was shaved and disinfected using povidone iodine. A skin incision of 1.0-2.0 cm was made on the dorsal surface of the calvarium and expanded by blunt dissection. An 8 mm critical size defect was made in the parietal bone between the occipital and frontal bones, taking care to leave the dura undisturbed (Figure 2, 3). The defect was created using an 8 mm Arruga trephine (Miltex, Bethpage, NY) and copious saline irrigation. After hemostasis was achieved the rats received implantation at random from one of the experimental groups. Wound clips were placed for primary closure of the surgical site. The rats were placed two to a cage for healing and were allowed water and food *ad libitum*. During healing no clinically observable negative effects were noted for the rats.

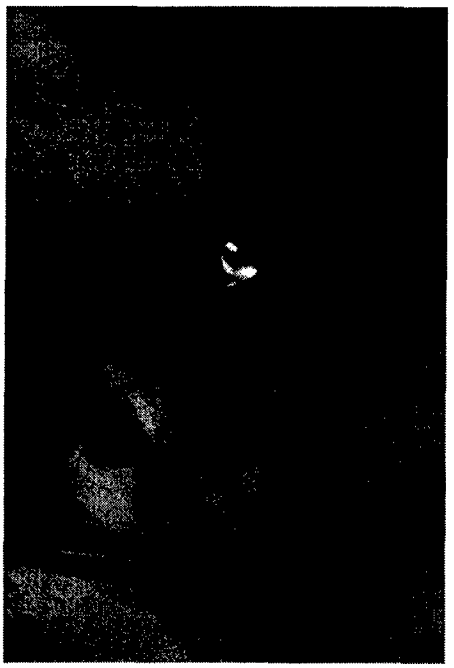
Figure 2 A and B: Formation of 8 mm Critical Size Defect

A skin incision of 1.0-2.0 cm was made on the dorsal surface of the calvarium and expanded by blunt dissection. An 8 mm critical size defect was made in the parietal bone between the frontal and occipital bone, taking care to leave the dura undisturbed.

Panel A: Shows the 8 mm trephine used to create the defect.

Panel B: Shows the critical size defect with the underlying dura intact.

A.



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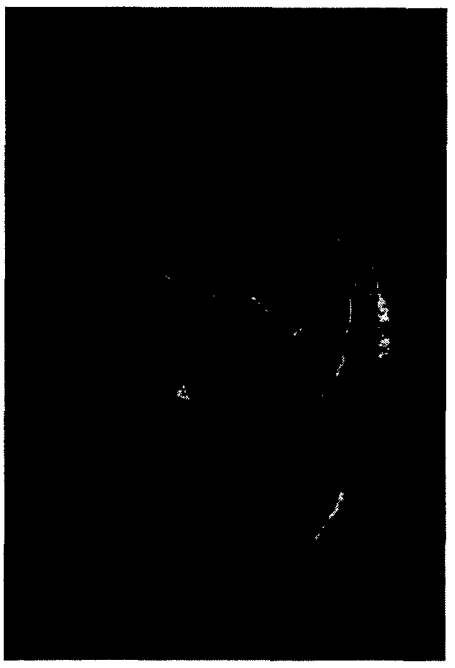
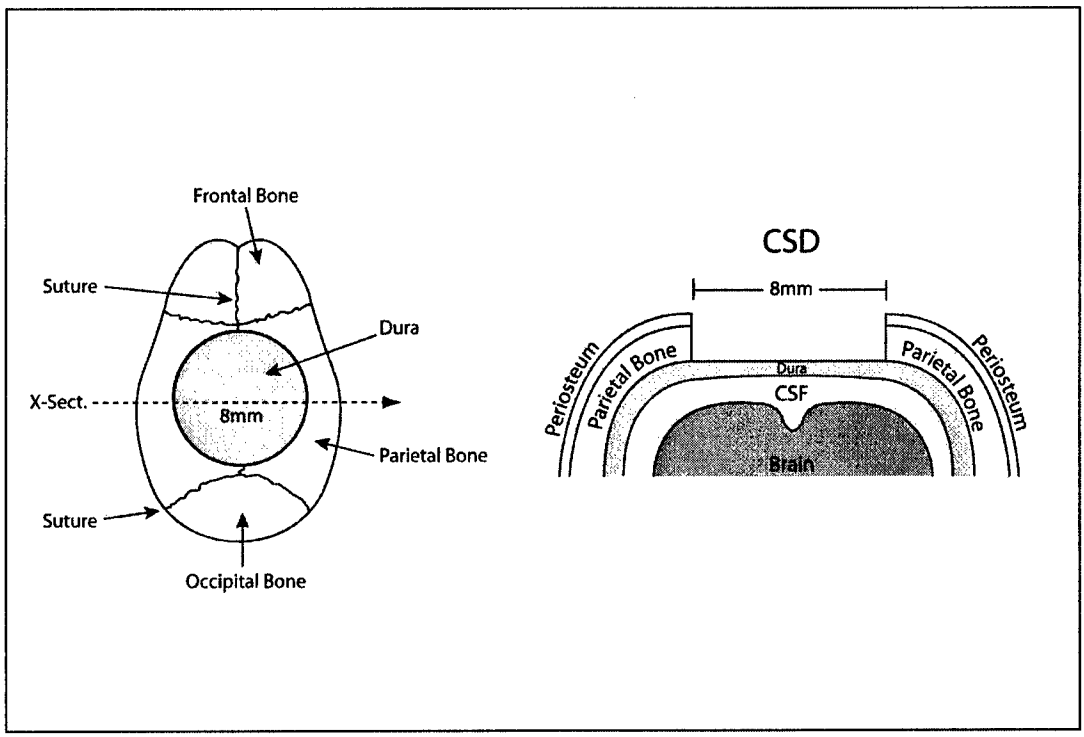


Figure 3: Schematic of Critical Size Defect

An 8 mm critical size defect was made in the parietal bone between the frontal and occipital bone.


Frontal Bone
Suture
Dura
X-Sect.
8mm
Parietal Bone
Suture
Occipital Bone
CSD
8mm
Periosteum
Parietal Bone
Dura
CSF
Brain
Parietal Bone
Periosteum

5. Histological Evaluation

The rats were euthanized by asphyxiation with carbon dioxide at two and eight weeks. Tissue was recovered from the implant site and fixed in 10% formalin. The samples were then decalcified in Surgipath (Richmond, IL) decalcifier II solution. The samples were sectioned through the middle of the defect and placed face down on the slide and embedded in paraffin. The samples were then sectioned at 3-4 μm each, and stained with hemotoxylin and eosin. One section from each level was evaluated and the data are pooled for each defect site.

The entire section was evaluated for presence of DFDBA particles, dentin matrix particles, and new bone using a scoring system (Table 2) as previously described (Boyan *et al* 2000). Briefly sections with no new bone formation or carrier were scored 0; sections with no new bone formation but the DFDBA or DDM was present, were scored 1; sections with new bone in one site <40% of surface area were scored 2; sections with new bone in more than one site covering >40% but <70% were scored 3; a section with new bone formation in more than one site covering >70% of the surface area were scored 4. There was one examiner for all the slides who was blinded to the test groups. When no DFDBA or DDM was present in the section the new bone formation was scored and the number received a star. This test group number was then checked against the master key. If the group contained EMD alone the score for the new bone formation was recorded. If the test group was to contain DFDBA or DDM the score resulted in a zero and was not included in the statistical analysis.

D. Statistical Analysis

RIDIT (relative to an identified distribution) analysis was used to statistically analyze the bone score data (Fleiss 1973). RIDIT analysis is appropriate for sample data that is qualitatively ordered because it takes advantage of ordering that exists but does not require a gradient in proportions. In RIDIT analysis, the discrete categories represent intervals on some unobservable distribution. The RIDIT analysis uses a reference group. In the current study the reference group was propylene glycol alone. The reference group has a mean RIDIT value of 0.5. All other groups are compared to the reference group. The resulting mean RIDIT value of a group that is 0.5 indicates that there is no greater probability of selecting a sample out of that group with more new bone formation than out of the reference group. A RIDIT value greater than 0.5, indicates that group has a greater probability of the outcome, which is more new bone formation. A RIDIT value less than 0.5 indicates a lesser probability of finding greater new bone formation in that group than in the reference group. A change of 0.1 RIDIT unit would indicate a 10% variable probability of either an increase or decrease in bone formation compared to the reference group. P values ≤ 0.05 were considered significant.

Although RIDIT analysis is appropriate for qualitatively ordered categories, it is less powerful at small sample sizes. Therefore, descriptive statistics were also determined for the bone score data. The data were analyzed using a 3-way ANOVA followed by pair-wise comparison of the means. The three factors were healing time, diluent, and carrier. Analysis included all interactions of the factors, and for pair-wise comparison of the means, the effect of each factor was adjusted for the effects of the other factors (type III).

In addition to analysis of the raw data, a standard square root transformation of the data was performed. This was done because of the small number of ordered categories, as well as to assure that the standard assumptions for valid analysis were satisfied, that the data were normally distributed and the variances equal.

Table 2: Scoring of New Bone Formation

Sections were evaluated with a scoring system as previously described (Boyan *et al* 2000).

Bone formation score

0= no new bone or carrier

1= no new bone with carrier

2= new bone in one site <40%

3= new bone >40% but <70%

4= new bone >70%

E. Retention time of Enamel Matrix Derivative

1. Radiolabeled Enamel Matrix Derivative

EMD was radioactively labeled with ^{125}I (Rosenberg, Teare 1977). Thirty milligrams of EMD were dissolved in 3 ml of 0.01% acetic acid and allowed to equilibrate at room temperature. This stock solution of EMD was further diluted to a final concentration of 1mg/ml in cold phosphate buffered saline, pH 7.2. Carrier free $Na^{125}I$ obtained from PerkinElmer Life Sciences (Billerica, MA) was diluted to 5 mCi (185 MBq) / 25 µl with PBS, pH 7.2. The iodination was performed at room temperature in a 12 x 75 mm glass tube by the sequential addition of 5 µl $Na^{125}I$, 25 µl EMD, and 5 µl chloramine T (5 mg/ml in PBS, pH 7.2). After 30 seconds the reaction was stopped by the addition of 5 µl sodium metabisulfite (10 mg/ml) and 460 µl of 30% acetonitrile-0.15 M sodium chloride. For separation of the iodinated protein from unreacted iodide, the entire reaction mixture was applied to a PD-10 column previously equilibrated with 30% acetonitrile-0.15 M sodium chloride. Elution was carried out using the same solution and fractions of 1 ml / tube were collected. The fractions were assayed for radioactivity in a Beckman Gamma 5500 gamma scintillation counter, and the iodination product eluted in fractions 6 through 9 were used for the experiments. These fractions were pooled and assayed for activity /volume in a Radix Dose Calibrator (Houston, TX). Instant TLC, performed using SG-silica gel strips (Gelman Instrument Company, Ann Arbor, MI; currently available from VWR International) determined the per cent of free iodine to be less than 10% (Rosenberg, Teare 1977). The entire iodination procedure was repeated as just described with more EMD, the iodination products from each procedure pooled, and

the volume reduced using a stream of nitrogen. The radioactive concentration of the final solution was 30 μCi / 25 μl.

2. Implant Material Preparation

On the day of the surgery DFDBA (10 mg) or DDM (10 mg) was mixed with 25 μl of the reconstituted ^{125}I radiolabeled EMD (30 μCi / 25 μl).

3. Experimental groups

The rats were divided into three groups of 6 rats each: 1) radiolabeled EMD 2) DFDBA and radiolabeled EMD 3) DDM and radiolabeled EMD.

4. Surgical Protocol

The rats were anesthetized with isoflurane gas (4 L/min) with oxygen (2 L/min). Gas alone was used to achieve a short recovery period. The surgical procedure was the same as Part I of the experiment. After hemostasis, 25 μl (30 μCi) of the ^{125}I radiolabeled EMD was added to the defect alone or combination with either 10 mg of DFDBA or 10 mg of DDM. Primary closure of the wound was achieved with 4-0 non-resorbable monofilament nylon sutures for primary closure. Two rats were placed to a cage for healing and were allowed water and food *ad libitum*. During healing no clinically observable negative effects were noted for the rats.

5. Gamma Scintigraphy

Scintigraphic images were acquired immediately after implantation (0 hour), at 4 hours, and at 1, 2, 3, 6, 13, and 20 days. The rats were imaged under isoflurane gas anesthesia as described above. Imaging was performed with a Picker SX-300 Spect Gamma Camera (Picker Instruments, Cleveland, OH). Before imaging, the camera was calibrated using ^{57}Cobalt. Also, an extrinsic flood quality control image was obtained with a sheet of uniformly distributed ^{57}Cobalt. Because the energy of ^{125}I is low for this camera, the collimator was removed and the camera shielded with a 5 mm thick, solid lead sheet except for a 1.0 cm hole that was placed directly over the area of the defect. The camera head was centered over the hole and lowered to within 0.5 cm of the lead shield. Image acquisition was performed with a MEDASYS Pinnacle computer work station (MEDASYS, Miami, FL) interfaced to the SX-300 camera. Background acquisitions along with ^{125}I standards were taken daily prior to the animal acquisitions. Control images were obtained by spotting 25 µl containing 30 µCi of ^{125}I labeled EMD on 8 mm disks of filter paper. The dried disks were placed on the lid of a small Petri dish and positioned directly under the hole in the lead shield for image acquisition. The control disks were imaged for each time point in the study. A 10 minute acquisition period was used to obtain counts that were at least 10 times greater than background. Image analysis of uniform regions of interest containing identical pixel numbers from the acquired images was performed using OSIRIS software (University Hospital of Geneva, Switzerland). Retention of ^{125}I at the defect site was determined and corrected for radioactive decay.

F. Statistical Analysis

The ^{125}I retention data were analyzed with multiple comparison analysis, a type of ANOVA, using a Tukey's procedure as the post-hoc test. Significance was determined at the $p \leq 0.05$ level.

RESULTS

A. Analysis of New Bone Formation

The surgical procedures and healing were as expected with a couple of exceptions. First, there were animal deaths, thus decreasing the groups' size, traced to defective anesthetic cocktail. Second, there were differences in the surgical skill on the part of the surgeon which may have contributed to the variability of the results. The observed mean RIDIT values for the treatment groups are displayed in Tables 3, 4 and Figure 4.

Two week observations:

At two weeks, RIDIT analysis indicated that there were no statistically significant differences between the reference group (propylene glycol alone) and the treatment groups (Table 3).

EMD alone: One mg of EMD resulted in the same mean RIDIT value as the reference group. Placement of 3 mg and 9 mg of EMD resulted in 0.2 and 0.3 mean RIDIT value respectively, which indicated there was a lower probability of finding new bone formation in these groups than the reference group.

DFDBA group: DFDBA alone groups, with and without diluent, resulted in the same mean RIDIT value as the reference group (0.5). When EMD was added to DFDBA (1 mg, 3 mg, 9 mg) the resulting mean RIDIT values were less than the reference group

(0.2, 0.3, and 0.3 respectively). This indicated that there was a reduced probability of finding more new bone formation in these groups than the reference group.

Table 3*:* New Bone Formation at 2 weeks

The mean RIDIT values are displayed for each group as well as the number of animals per group. The reference group has a mean RIDIT value of 0.5. None of the groups were statistically different at two weeks ($p \leq 0.05$ statistically significant).

Group	Mean RIDIT	Number of rats in group
Diluent+0 mg EMD	0.5	6
Diluent+1 mg EMD	0.5	5
Diluent+3 mg EMD	0.2	6
Diluent+9 mg EMD	0.3	6
DFDBA+0 mg EMD	0.5	9
DFDBA+1 mg EMD	0.2	6
DFDBA+3 mg EMD	0.3	6
DFDBA+9 mg EMD	0.3	6
DDM+0 mg EMD	0.4	8
DDM+1 mg EMD	0.4	7
DDM+3 mg EMD	0.5	6
DDM+9 mg EMD	0.3	6
DDM without diluent	0.3	8
DFDBA without diluent	0.5	8

DDM group: The dentin matrix alone groups, with and without diluent, resulted in a reduced RIDIT value (0.4 and 0.3 respectively) compared to the reference group. The dentin matrix group with the addition of 1 and 9 mg of EMD also had a reduced RIDIT value (0.4 and 0.3 respectively) compared to the reference group. Dentin matrix particles mixed with 3 mg of EMD resulted in the same RIDIT value (0.5) as the reference group, indicating that there was an equal probability of finding new bone formation in either group.

Eight week observations:

At eight weeks (Table 4), the RIDIT analysis indicated that there was more bone formation in all the treatment groups compared to the reference group. However, with the exception of 3 mg EMD in combination with the DDM particles ($p \leq 0.05$), these values did not reach statistical significance.

EMD alone group: At eight weeks the mean RIDIT value of the 0 mg EMD group was 0.3. As the concentration of EMD increased from 1 mg EMD to 9 mg EMD there was a trend for increased bone formation (1 mg EMD=0.5; 3 mg EMD=0.6; 9 mg EMD=0.7). These were not statistically significant differences.

DFDBA group: The mean RIDIT values for DFDBA with diluent (DFDBA+0 mg EMD) and 1 mg EMD + DFDBA was 0.5, indicating that the amount of new bone formation was similar to the reference group. Both 3 mg and 9 mg EMD experienced a trend toward increased new bone formation with the same mean RIDIT value of 0.6.

DFDBA alone also received a mean RIDIT value of 0.6. There were no statistically significant changes in new bone formation in any of these treatment groups.

DDM group: All treatment groups showed a mean RIDIT value greater than the reference group. DDM with and without diluent, 1 mg and 9 mg EMD had a mean RIDIT value of 0.6. Three mg EMD in combination with DDM particles resulted in a mean RIDIT value of 0.8. This was the only value to achieve statistical significance ($p \leq 0.05$).

Hisotological analysis demonstrated little to no new bone formation noted away from the margin of the defect in the diluent (propylene glycol) alone groups. In the diluent groups soft tissue filled the majority of the defect. At eight weeks small islands of new bone were noted in select specimens (Figure 2). Histologically the bone formation response in the two carrier groups appeared differently. Interestingly, unlike the DFDBA particles, the DDM particles were encapsulated with fibrous soft tissue and were not in intimate contact with the new bone formation. Encapsulation of the DDM particles was not expected, but it did not seem to affect the bone formation response. The separation of the DDM particles from the surrounding fibrous soft tissue may be a processing artifact (Figure 11, 12). The appearance of the new bone formed in contact with DFDBA particles, appeared similar to when DFDBA is used in the ectopic model. This new bone was in intimate contact with the DFDBA particles (Figures 13, 14). EMD alone at all concentrations was slightly more osteoinductive than either DFDBA or DDM particles alone. At eight weeks sites treated with EMD showed increased amount and maturation of new bone formation (Figures 7, 9). Of note, cartilage like formation was observed at all concentrations of EMD alone at two weeks (Figures 6, 8, 10).

Table 4: New Bone Formation at 8 weeks

The mean RIDIT values are displayed for each group as well as the number of animals per group. The reference group has a mean RIDIT value of 0.5. Note 3 mg EMD in combination with DDM particles resulted in a mean RIDIT value of 0.8. This was considered statistically significant ($p \leq 0.05$ statistically significant).

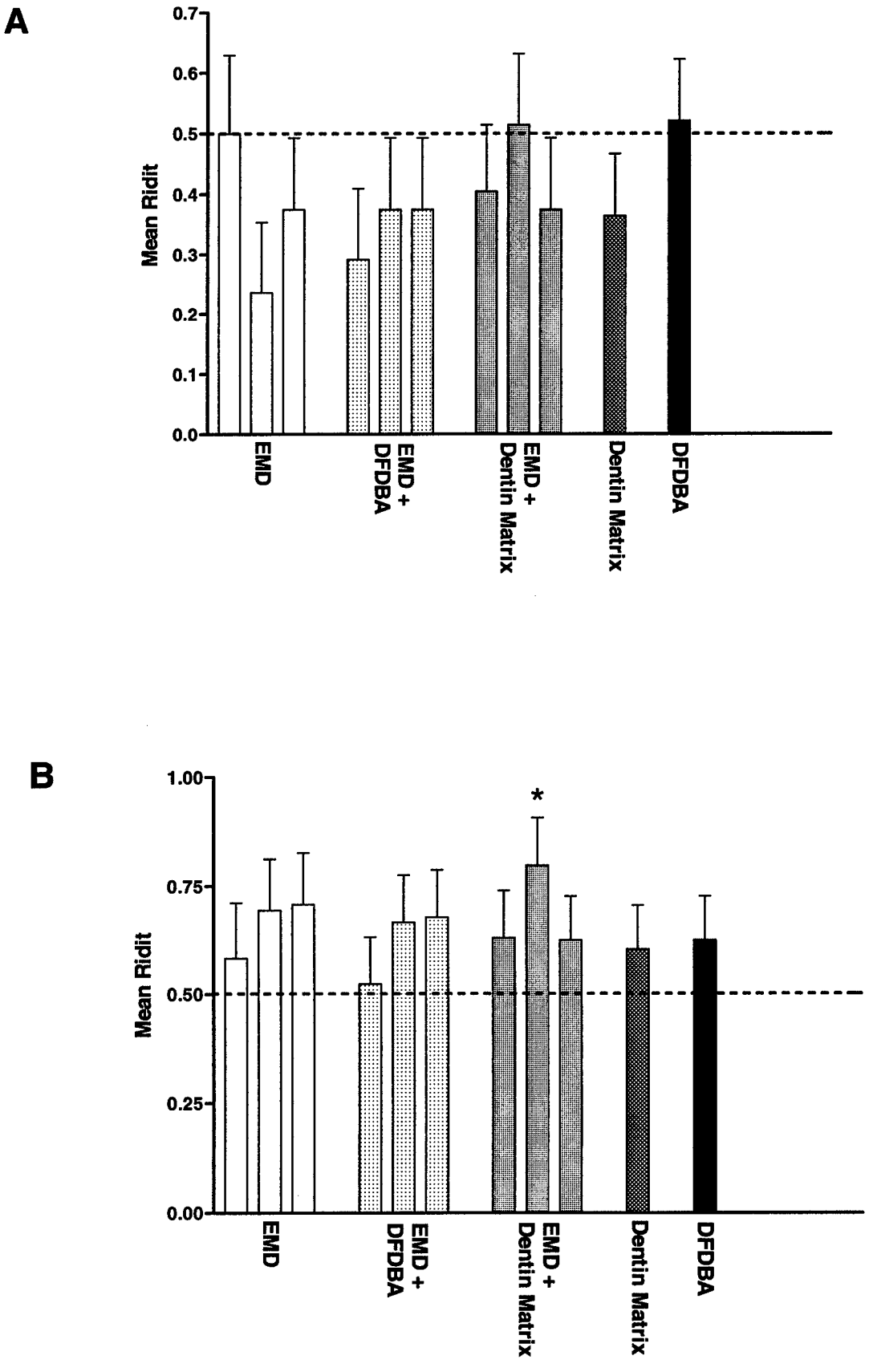
Group	Mean RIDIT	Number of rats in group	P value
Diluent+0 mg EMD	0.3	4	0.3
Diluent+1 mg EMD	0.5	5	0.5
Diluent+3 mg EMD	0.6	6	0.1
Diluent+9 mg EMD	0.7	6	0.07
DFDBA+0 mg EMD	0.5	8	0.7
DFDBA+1 mg EMD	0.5	7	0.8
DFDBA+3 mg EMD	0.6	7	0.1
DFDBA+9 mg EMD	0.6	7	0.1
DDM+0 mg EMD	0.6	8	0.3
DDM+1 mg EMD	0.6	7	0.23
DDM+3 mg EMD	0.8	7	0.0069*
DDM+9 mg EMD	0.6	8	0.23
DDM without diluent	0.6	8	0.3
DFDBA without diluent	0.6	8	0.23

Figure 4: RIDIT Analysis of New Bone Formation at 2 and 8 weeks

The bars in each group indicate 1 mg, 3 mg, and 9 mg concentrations of EMD.

Panel A: Illustrated are the mean RIDIT values at 2 weeks for each treatment group in addition to the various concentrations of EMD. The dotted line indicates the reference group (diluent alone). None of the treatment groups are significantly above the reference group.

Panel B: Illustrated are the mean RIDIT values at 8 weeks for each treatment group in addition to the various concentrations of EMD. The dotted line indicates the reference group (diluent alone). 3 mg EMD in combination with DDM particles mean RIDIT value was 0.8. The asterisk indicates statistical significant different at $p \leq 0.05$.


A
Mean Ridit
0.7
0.6
0.5
0.4
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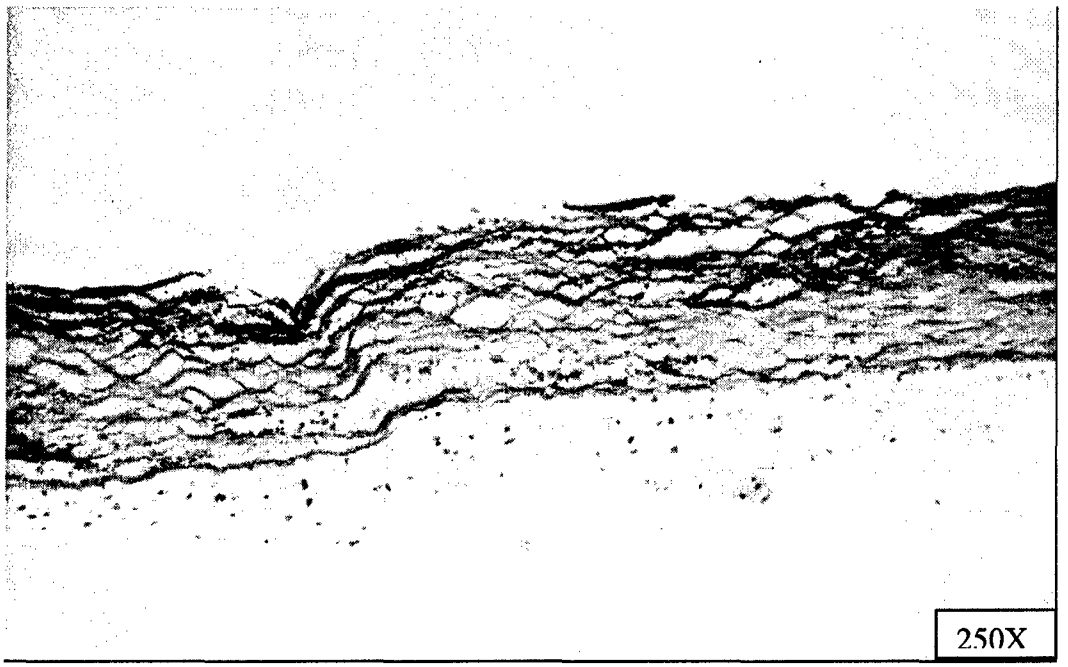
EMD
EMD + DFDBA
EMD + Dentin Matrix
Dentin Matrix
DFDBA
B
Mean Ridit
1.00
0.75
0.50
0.25
0.00
*
EMD
EMD + DFDBA
EMD + Dentin Matrix
Dentin Matrix
DFDBA

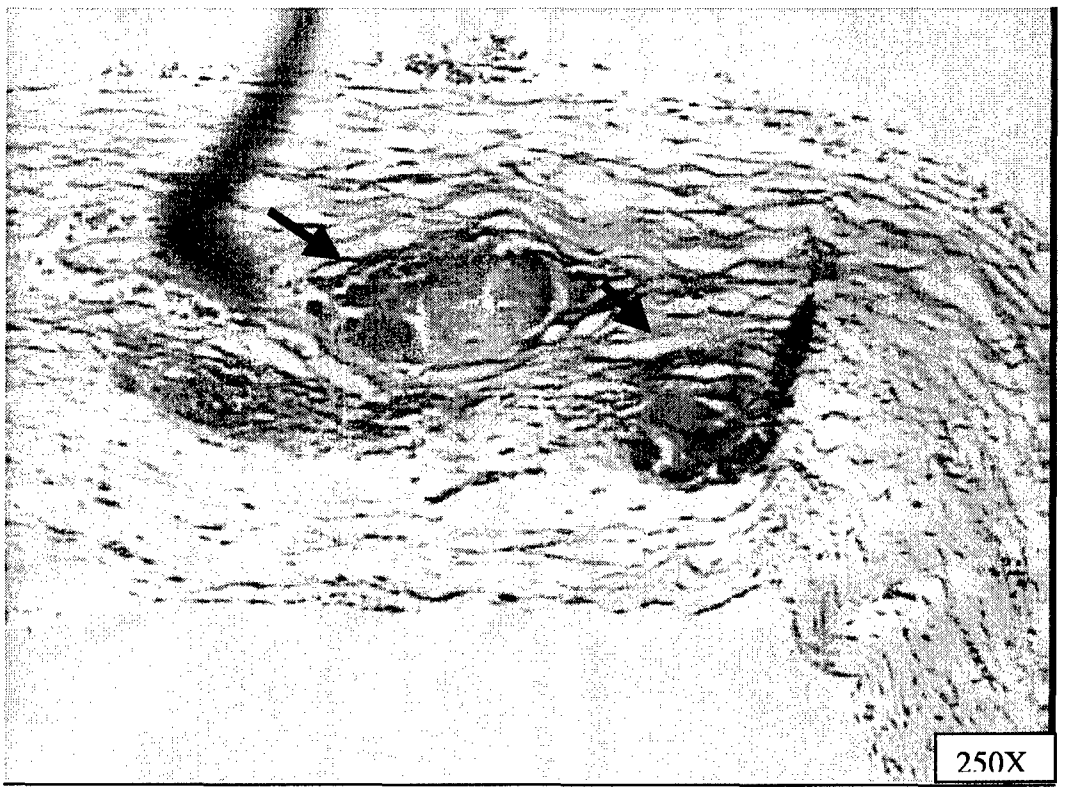
Figure 5: Histological Slide of Diluent at 2 and 8 weeks

Slide A: Diluent alone at two weeks.

Slide B: Diluent alone at eight weeks. Both groups had little to no new bone formation. The majority of the defects were filled with fibrous connective tissue. At eight weeks there were small islands of bone present in some of the specimens (arrows pointing to new bone).



A.



B.

Figure 6: Histological Slide of 1 mg EMD at 2 weeks

Arrows indicate observed cartilage formation in defects treated with 1 mg of EMD. This may suggest endochondral pathway for bone formation.

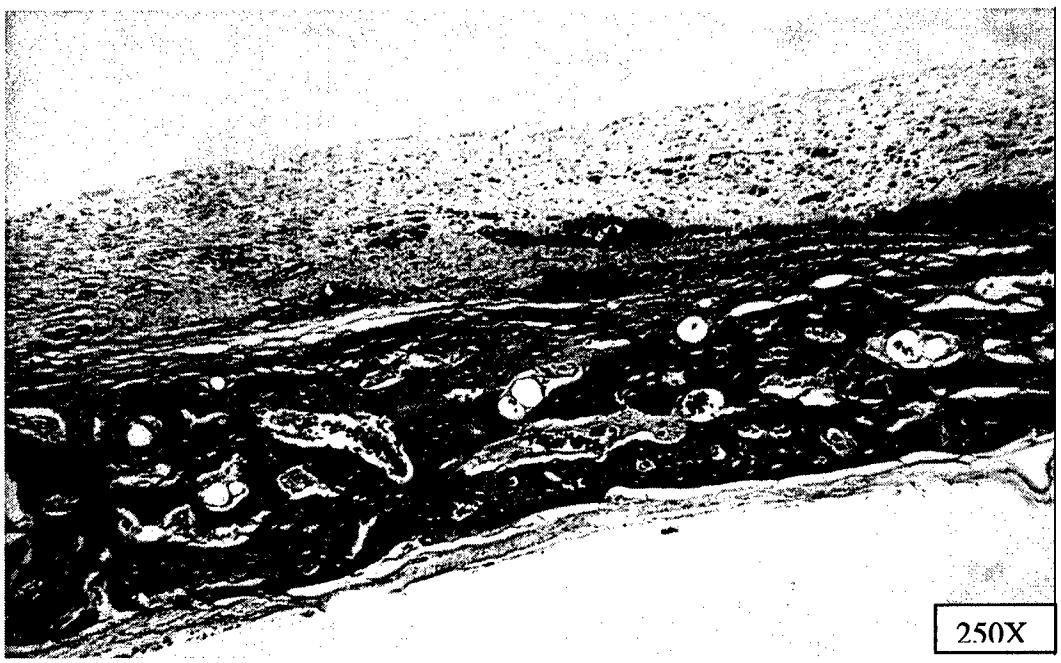

250X

Figure 7: Histological Slide of 1 mg EMD at 8 weeks

At eight weeks sites treated with 1 mg EMD showed an increase in amount and maturation of new bone formation compared to the two week specimens (arrow indicating new bone formation).

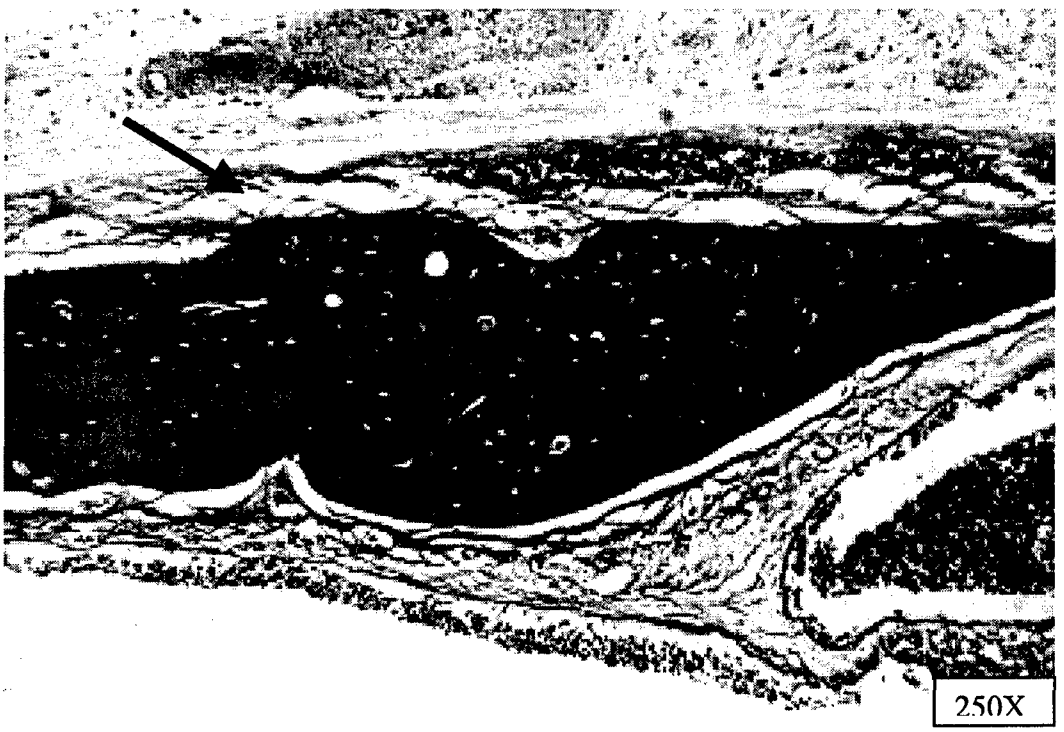
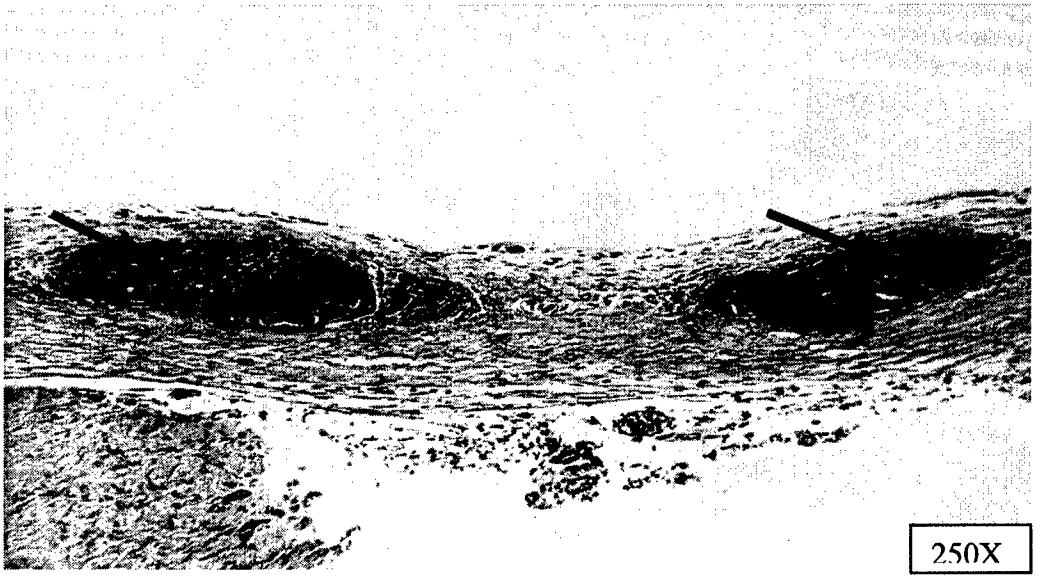

250X

Figure 8: Histological Slide of 3 mg EMD at 2 weeks

Arrows indicate observed cartilage formation in defects treated with 3 mg of EMD. This may suggest endochondral pathway for bone formation.



250X

Figure 9: Histological Slide of 3 mg EMD at 8 weeks

At eight weeks sites treated with EMD showed an increase in the amount and maturation of new bone formation compared to the two week specimens (arrow indicating new bone formation).

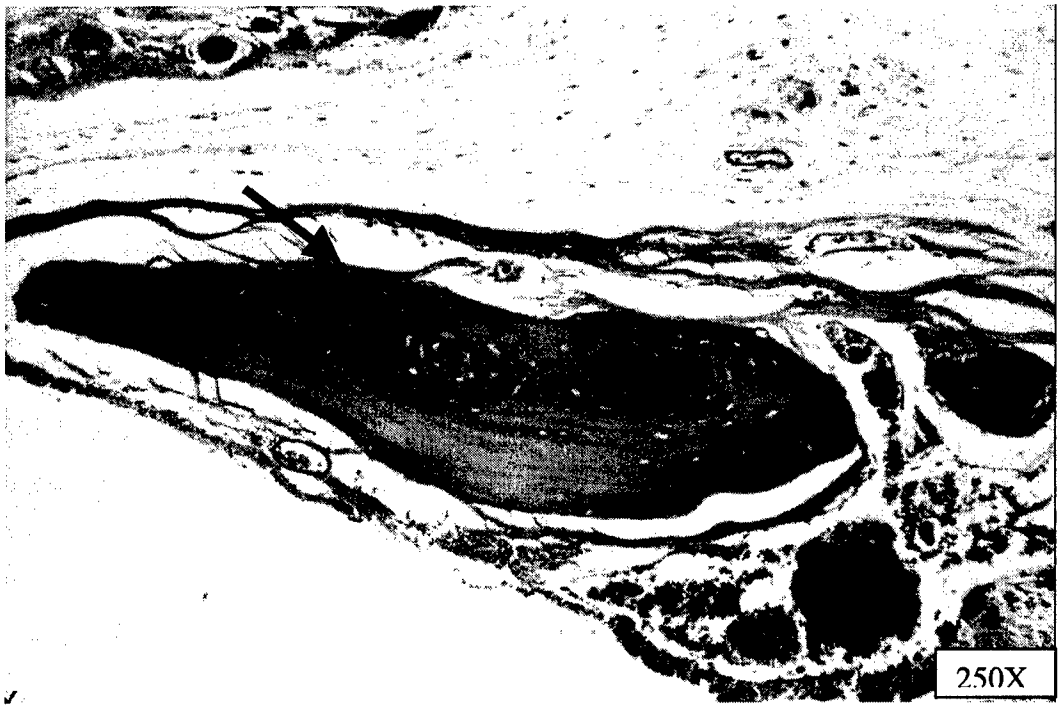
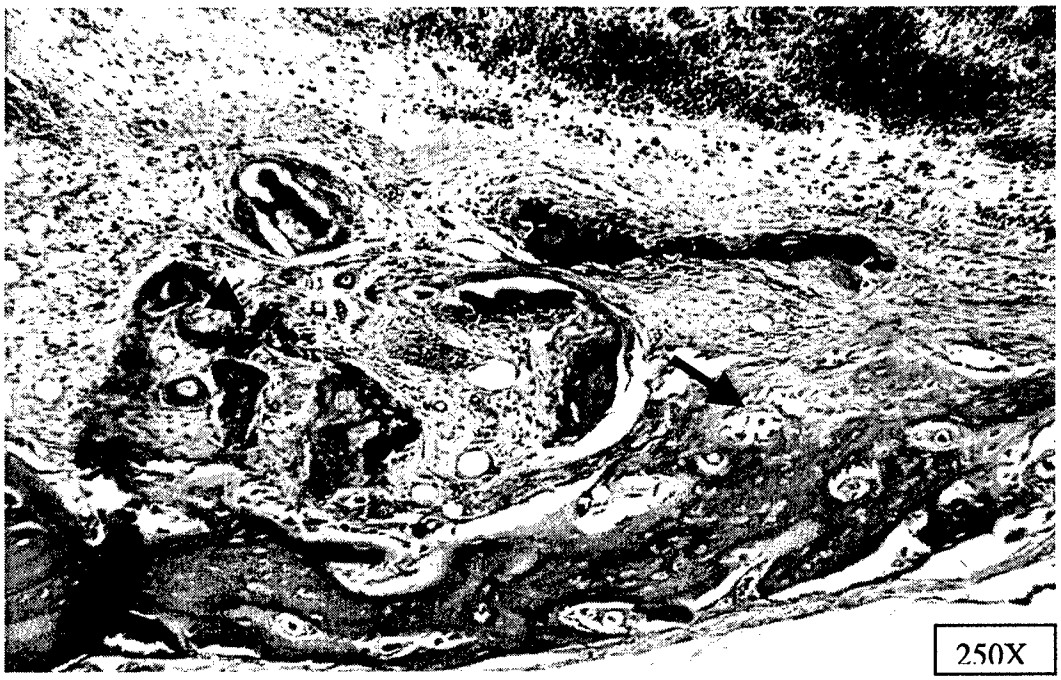

250X

Figure 10: Histological Slide of 9 mg EMD at 2 weeks

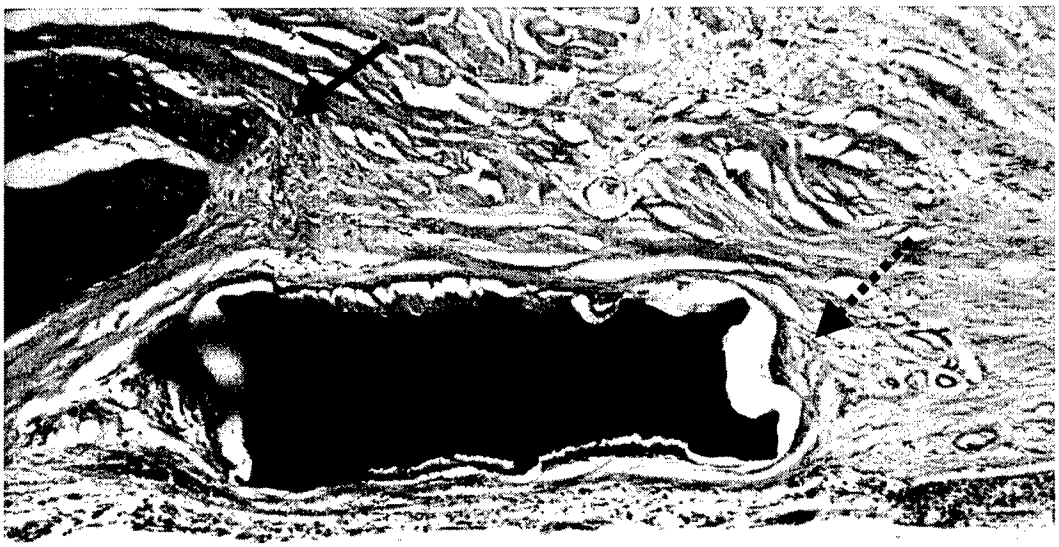
Arrows indicate observed cartilage and bone formation in defects treated with 9 mg of EMD. This may suggest endochondral pathway for bone formation.



250X

Figure 11: Histological Slide of DDM Particles + Diluent at 2 weeks

Fibrous soft tissue appears to have encapsulated the DDM particle. The dotted arrow indicates the space between the DDM particle and soft tissue. This may have been a processing artifact. Solid arrow points to new bone formation coming from the edge of the defect. This was not scored as new bone formation from the DDM particle.



250X

Figure 12: Histological Slide of DDM Particle + 9 mg EMD at 8 weeks

The solid arrow indicates new bone formation. Both lamellar and woven bone are present in the new bone formation. The new bone is adjacent to the dura side of the defect and bridges the majority of the defect. Note the bone is not in direct contact with the DDM particle (dotted arrow). Soft tissue appears to have encapsulated the DDM particle. The space between the DDM particle and soft tissue may be a processing artifact.

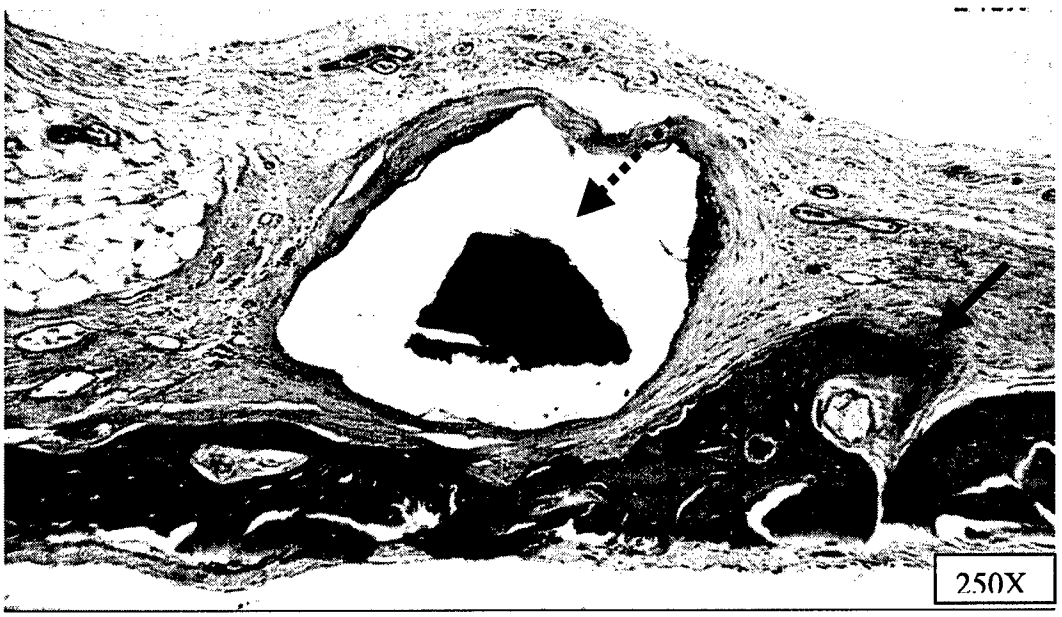

250X

Figure 13: Histological Slide of DFDBA Particles + Diluent at 2 weeks

DFDBA with diluent (propylene glycol) at two weeks, showed new bone formation surrounding the DFDBA particles. Solid arrows indicate new bone formation. Note the intimate contact with the DFDBA particle (dotted arrows).

250X

Figure 14: Histological Slide of DFDBA Particles + 3 mg EMD at 8 weeks

DFDBA with the addition of 3 mg EMD showed new bone formation surrounding the DFDBA particles. Solid arrows indicate new bone formation. Note the intimate contact with the DFDBA particle (dotted arrows) and the ruffled border at the lower portion of the larger DFDBA particle.


250X

B. Retention time of EMD

^{125}I labeled EMD disappeared rapidly from the defect (Figure 15). After four hours 50% of 30μCi/ 25 μl EMD was left in the defect. At 24 hours 20% of the total amount of EMD placed in the defect remained. Most of the disappearance of EMD occurred in the first 24 hours. At day 20 there was 4% of the total amount of EMD placed in the defect. Neither DFDBA nor DDM increased the retention time of EMD in the defect compared to EMD in the propylene glycol alone. There was a trend for EMD with DFDBA to be retained slightly longer than EMD with propylene glycol alone in the defect, though this was not statistically significant ($p \leq 0.05$).

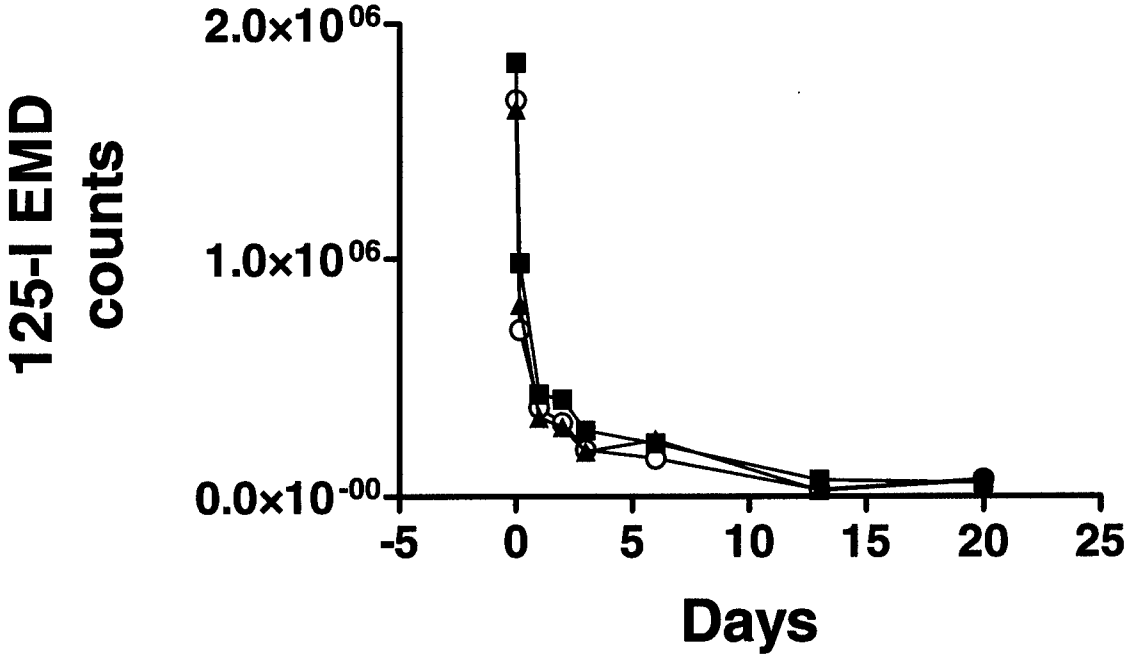
Figure 15: ^{125}I EMD Count from Critical Size Defect

At four hours the defect contained 50% of the total amount of EMD. The most significant decrease occurred within the first 24 hours (80% of the total EMD). At day 20 there was 4% of the total amount of EMD left in the defect. There were no statistically significant differences between the groups at individual time periods. Significant differences were between the early time points 0 hour vs. 4 hours and 4 hours vs. 24 hours at $p \leq 0.05$.

○ EMD (25 µl)

▲ EMD (25 µl) + DDM (10 mg)

■ EMD (25 µl) + DFDBA (10 mg)


125-I EMD counts
2.0×10 06
1.0×10 06
0.0×10 -00
-5
0
5
10
15
20
25
Days

DISCUSSION

The critical size defect model allows for study of the bone inducing properties of EMD, while evaluating the effects of two space maintaining carriers. The data demonstrated that while DFDBA and DDM particles do not enhance new bone formation with EMD over EMD alone, they do not interfere with it either. The data refutes the hypothesis that the use of space maintaining carriers with EMD increases new bone formation in the rat calvaria.

A. Analysis of new bone formation

The critical size defect in the rat calvarium is a proven model for the demonstration of bone regeneration (Schmitz, Hollinger 1986). EMD has been shown to enhance new attachment in periodontal defects (Heijl 1997). Boyan *et al* (2000) prepared suggested that EMD is an osteopromotive factor because of its ability to enhance new bone formation when used in combination with active DFDBA in an ectopic bone formation model. DFDBA has been shown to contain bone morphogenic proteins 2, 4, and 7 (Shigeyama *et al* 1995). Similarly demineralized dentin matrix particles have been shown to contain bone morphogenic proteins, as well as TGF-β, and IGF-I and –II (Finkelman *et al* 1990). Neither the DFDBA nor the DDM particles used in this study had been assayed for osteoinductivity by their respective manufacturer. In the present study similar amounts of new bone formation were observed at eight weeks for the DFDBA

and DDM particles when implanted alone in the critical size defect. Although the amounts of new bone were greater in the DFDBA and DDM group compared to the amount of new bone in the reference group (propylene glycol), the results were not statistically different due to the variability. With a larger sample size statistical significance would have been achieved. Group sizes for this experiment were chosen based on results of the ectopic induction protocol used in Boyan *et al* (2000). Because some animals did not survive to 8 weeks, some group sizes were reduced, thereby affecting the statistical procedures. Also, the degree of variability observed in the critical size defect model compared to the ectopic model, in which a more robust response has been noted, indicates that larger sample sizes will be required to achieve statistical significance. With a standard deviation of 0.25, which was used in our study, we would need 18 animals per group to show a statistical difference between the data means. The results of this study do not agree with Boyan *et al* (2000) findings that EMD increased osteopromotion with DFDBA. The ectopic model is a closed system that contains inherent wound stability from the surrounding muscles. The critical size defect in rat calvarium is a more stringent model for regeneration due to the ability of the rats to disturb the surgical site. In addition, in the previously mentioned study the results demonstrated that EMD did not enhance inactive DFDBA. The DFDBA in this study may not have been biologically active.

Histologically, the bone formation response in the two groups appeared different. While the DFDBA particles were in intimate contact with the new bone formation, the DDM particles were encapsulated with fibrous connective tissue from the new bone formation. The appearance of new bone formed in intimate contact with DFDBA

particles is similar to that observed in the ectopic model as well. Encapsulation of the DDM particles was not expected, but it did not seem to affect the bone formation response. This lack of contact with new bone suggests that the DDM particles were osteoinductive in nature or was an artifact.

EMD alone was slightly more osteoinductive at eight weeks than were DFDBA or DDM particles alone or in combination with EMD, particularly at the higher concentrations used. Again statistical significance was not achieved due to variability and sample size. Of note was the cartilage-like formation was observed at all concentrations of EMD alone groups at two weeks. This suggests that EMD may be promoting osseous regeneration through an endochondral pathway.

This may be beguiling significant, because cartilage formation typically is not often observed during bone formation in the critical size defect model (Wang, Glimcher 1999). Interestingly, cartilage formation was rarely observed in the experimental groups where EMD was combined with the DFDBA or DDM particles. Further studies are needed to assess the significance of increased cartilage-like formation with EMD alone and exact pathway of bone formation with EMD.

Most new bone formation observed in this study in the combination groups occurred at or near the dural margin in the defect. This is consistent with the pattern of healing observed in the literature for calvarial defects (Ozerdem *et al* 2003). While both the periosteum and the dura mater have been shown to induce new bone formation, unlike the periosteum of bones in the appendicular skeleton, the calvarial periosteum contributes minimally to bone healing compared to the dura mater (Gosain *et al* 2002). The dura mater contains cells, which can differentiate into osteoblasts. Cytokines

secreted at the wound site or liberated from implanted graft material can induce cellular differentiation, which results in repair of the osseous defect (Wang, Glimcher 1999). Most evidence points to a mechanism of cranial defect healing which utilizes primarily by autocrine and paracrine signaling from the underlying dura mater (Ozerdem *et al* 2003). Therefore, the variability in new bone formation observed in the present study may be explained by the quality and/or quantity of the dura mater underlying the critical size defect. While care was taken to avoid damage to the dura during surgery, one surgeon was more experienced than the other with the critical size defect model system. Thus, the dura may have sustained more damage in animals operated on by the less experienced surgeon.

The RIDIT analysis used in this study is a relatively uncommon statistical analysis. Nevertheless, it is a useful statistical method when random distributions of items or responses with a relative correlation are under study. In the RIDIT analysis, a reference group to which all other groups are compared is identified. The reference group in this study was the group with diluent alone (propylene glycol). This group was chosen because it was considered the group most likely to have the least new bone formation response. The reference group has a mean RIDIT that always calculates to a value of 0.5. At two weeks none of the treatment groups had a higher mean RIDIT value than the reference group. This indicates that there was no more probability of finding new bone formation in the treatment groups than the reference group. Bone formation usually requires resorption prior to the formation of new bone. At this early two weeks stage it is possible that there was insufficient time for the resorptive and formative processes to be completed. At eight weeks the mean RIDIT values for all groups were greater than 0.5,

indicating that the probability of finding new bone in these groups was greater than that of the diluent alone reference group. Unfortunately, only 3 mg EMD combined with the DDM particles had a statistically significant higher mean RIDIT value than the reference group (0.8, $p \leq 0.05$). This indicates that there was a greater probability of finding new bone formation in this group compared to the reference group. The lack of significance in the face of this trend in the data again highlights the variability observed in the critical size defect model and the requirement for a greater experimental sample size.

Although RIDIT analysis is appropriate for qualitatively ordered categories, it is less powerful at small sample sizes. Therefore, descriptive statistics were also determined for the bone score data. The data were analyzed using a 3-way ANOVA followed by pair-wise comparison of the means. The data was also analyzed following a standard square root transformation of the data. These analyses were in agreement with the results obtained by RIDIT analysis.

B. Retention time of EMD

Gamma scintigraphy is utilized to follow the distribution of radiolabled compounds in live animals. The gamma radiation of ^{125}I is of low energy and difficult to detect using standard instrumentation available. By removing the collimator, the radioactive emissions were detectable. A lead plate was used in place of the collimator to limit the detection of background emission around the surgical site. A 1.0 cm hole cut into the lead and placed directly over the surgical site permitted detection of specific emission. It was determined that 10 minute acquisitions were sufficient to differentiate

between the signal at the surgical site and the background radiation. This time frame was sufficient to accumulate a signal at least 10 times that of the background.

The data from the present experiments did not support the hypothesis that DFDBA or DDM particles increased retention of EMD in the critical size defect. All groups displayed a significant decrease in radiation emission by 24 hours. On the first day, 80% of the EMD was lost from the defects. The physical half-life of ^{125}I is 60.14 days. Thus, the loss of the EMD from the defects could not be explained by the decay of ^{125}I. Instead the labeled EMD was physically or metabolically displaced from the surgical site. This study did not track the migration of the EMD. However, no clinically observable negative effects were noted for the rats.

Wound stability is an important aspect of regeneration. The critical size defects were 8 mm wide and 1 mm deep. The skin was primarily closed over the graft materials. Nevertheless, the rats could easily reach the surgical area and disrupt the surgical wound site while scratching at the staples or sutures. A membrane could possibly have provided the regenerative site more stability and protection but also could influence the results. Rapid displacement of EMD from the critical size defect site may have been a factor in the limited new bone formation observed. Particles of DFDBA and DDM were consistently observed during analysis of the defect site, indicating that these materials were in large part retained in the defect. This also suggests that EMD did not tightly bind to the materials.

In summary, this study indicated that DFDBA and DDM were not essential for new bone formation with EMD in the rat calvaria. While the DDM particles were suitable for space maintenance in the defect, their effects were not appreciably different from the

DFDBA and neither added significantly to bone formation over EMD alone. Further investigations using a membrane may improve the ability to detect any differences present between the treatment groups. Likewise, increasing the sample size in subsequent investigations will improve the ability of the model to detect significant differences in the combination materials as well.

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VITA

Kerri Font was born [redacted] in Des Moines, Iowa, daughter of [redacted] and [redacted]. She attended San Diego State University and graduated with a Batchelor of Science degree in Biology with magnum cum laude distinction. She worked one year at Scripps Clinic microbiology laboratory in San Diego, California after which she began dental training at the University of the Pacific in San Francisco, California.

While in dental school, she pursued periodontal research. She received Tau Kappa Omega award in 2001, Omicron Kappa Upsilon and The American Academy of Periodontology Student awards in 2002. In 2002 she was placed on the National Dean's List. She then received her Doctor of Dental Science in May 2002.

In the summer of 2002, she began a Postdoctoral Program in Periodontics at Lackland Air Force Base in conjunction with the University of Texas Health Science Center at San Antonio. In 2003 she was accepted into the Graduate School of Biomedical Sciences for the Masters of Science degree in Periodontics. After graduation in May 2005, she plans to practice periodontics in the Air Force at Ramstein AFB, Germany.

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